ADAPTATION OF MEMBRANE-BOUND ENZYMES TO ETHANOL

By

BENJAMIN FISHER DICKENS, JR.

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DEDICATION

To my wife, Bonnie, who stood by me through the years it took to achieve this goal; to my son, Brian, who has brightened my life; to my parents, to whom I owe more than I will ever be able to repay; this dissertation is dedicated to all of you.

ACKNOWLEDGMENTS

I would like to offer special thanks to Dr. L. O. Ingram, who as my major professor offered me the benefit of his knowledge and experience. I would also like to express my appreciation to Dr. E. M. Hoffmann and Dr. C. M. Allen Jr. for serving on my graduate committee and for their assistance during my graduate career. In addition, I am very grateful to the faculty of the Department of Microbiology and Cell Science who were always willing to share their time and resources.

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KEY TO ABBREVIATIONS

ATP: Adenosine Triphosphate

ATPase: Adenosine Triphosphatase

E_a: Energy of Activation

lac permease: Lactose Permease

ONPG: o-nitrophenol β-D-galactopyranoside

S. D.: Standard Deviation

16:0 : Palmitic acid

18:1 : Oleic acid

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of the University of Florida in Partial Fullfilment of the Requirements
for the Degree of Doctor of Philosophy

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Chairman: Lonnie O. Ingram
Major Department: Microbiology and Cell Science

Previous studies have shown that the proportion of unsaturated fatty acids increases in Escherichia coli during growth in the presence of ethanol. These lipid changes presumably represent an adaptive response to compensate for the direct physical effect of ethanol on the membrane lipid bilayer. The effects of growth in the presence of ethanol on two membrane-bound enzymes, the lactose permease and adenosine triphosphatase, were investigated. All but one of the properties of these membrane-bound enzymes, the activity of membrane-bound ATPase with Ca^{++} as a cofactor, were found to adapt to the presences of ethanol. The role of new protein synthesis and the ethanol-induced changes in the bulk lipid composition in the adaptation of membrane-bound enzymes to ethanol was also investigated.

Results of these investigations led to a model proposing that ethanol's effect on both membrane-bound enzymes and the adaptation of these enzymes to ethanol is mediated through the enzymes annular lipids.

INTRODUCTION

Living organisms display a remarkable ability to adapt their membranes to environmental changes such as temperature, by altering their lipid composition (Fulco, 1974). These lipid changes are thought to maintain the proper environment within the membrane for biological function (Sinensky, 1974). Ethanol has been shown to directly intercalate within biological membranes altering membrane organization (Hill and Bangham, 1975; Lee, 1977; and Seeman, 1972). Growth of *E. coli* in the presence of ethanol results in the synthesis of membrane lipids with an altered fatty acid composition, analogous to the changes observed following a decrease in growth temperature (Ingram, 1976).

The enzymes of fatty acid synthesis appear to be a major site for the regulation of lipid composition in *E. coli* (Cronan, 1975). Temperature-induced changes in the fatty acid composition are mediated in part through direct effects on these soluble enzymes (Cronan, 1975). Investigation of the mechanism of the ethanol-induced fatty acid changes indicate that the site of action is also the soluble fatty acid synthetase enzyme (Buttke and Ingram, 1978). This has recently been confirmed in our laboratory *in vivo*.

Ethanol has been shown to affect the activity of a variety of membrane-bound enzymes. Membrane-bound ATPases in eukaryotic

cells have been frequently studied (Grisham and Barnett, 1972; Mitjavila et al., 1976; Post et al., 1972; and Sun, 1976). Ethanol generally inhibits the oubain-sensitive (Na,K)-ATPase but has little effect on the activity of the Ca^{++}-ATPase. Transport systems have also been studied and are usually inhibited by ethanol in eukaryotic cells (Chang et al., 1967; Fox et al., 1978; Hoyumpa et al., 1977; Israel et al., 1968; and Worthington et al., 1978). In E. coli, alcohols of different chain lengths have opposite effects on the β-galactoside transport enzyme, the lac permease. Alcohols of chain lengths greater than four stimulate lac permease activity (Sullivan et al., 1974) while alcohols of shorter chain lengths are inhibitory (Fried and Novick, 1973). A similar differential effect of short and long chain alcohols has been reported on fatty acid composition (Ingram, 1976).

The fatty acid changes induced by ethanol in E. coli have been proposed as part of an adaptive response, compensating for the direct physical effects of ethanol (Ingram, 1976). The similarities between the lipid changes induced by ethanol to those resulting from a decrease in growth temperature suggest that both may be involved in the maintenance of the proper lipid environment for membrane function. In this paper, we have investigated the significance of ethanol-induced changes for membrane function in E. coli using two enzymes, ATPase and lac permease. The effects of ethanol on these enzymes were compared between cells grown in the presence and in the absence of ethanol.

MATERIALS AND METHODS

Lactose Permease

Escherichia coli K12 strain K1-221 (lac i^-), generously donated by Dr. R. P. Boyce (University of Florida, Gainesville, Florida, 32611), was used to examine the effect of ethanol on the lac permease. Cultures (250mL) were grown in mineral salts medium M63 (Miller, 1972) supplemented with succinic acid (5g/L), L-threonine (40mg/L), L-tyrosine (20mg/L), L-proline (30mg/L), L-histidine (22mg/L), uracil (40mg/L) and thiamine (20mg/L) in oversized culture tubes with continuous aeration or in 2-L flask with continuous shaking at either 30 °C or 37 °C. Lac permease activity was assayed as described by Fried and Novick (1973). Cells were harvested in exponential phase by centrifugation and resuspended in inhibitor buffer. Portions of this cell suspension were removed and supplemented with o-nitrophenol/β-D-galactopyranoside (ONPG). The reaction was stopped by the addition of calcium bicarbonate and the o-nitrophenol concentration determined spectrophotometrically at 420nm. Formaldehyde treated controls were used to correct for cryptic transport (Kepes, 1971).

In contrast to the above assay, the in vivo permease activity was determined in actively growing cells by supplementing 1mL samples of growing cultures with ONPG (1.85mM) and continuing to incubate under growth conditions for 3-7 minutes. Permease activity was measured as

o-nitrophenol produced using a formaldehyde control. Total β-galactosidase activity was measured by the procedure of Putman and Koch (1975). Cell numbers were estimated by turbimetric measurements.

ATPase

Escherichia coli K12 strain CSH-2, obtained from the Cold Spring Harbor Laboratory (Cold Spring Harbor, N. Y.), and a derivative of this strain blocked in fatty acid degradation (Buttke and Ingram, 1978), strain TB-4 (fad E^-), were used to examine the effects of ethanol on ATPase. Cultures (250mL) were grown in glucose-supplemented Luria broth (Luria and Delbruck, 1943) in 2-L flask with continuous agitation at either $30^{\circ}C$ or $37^{\circ}C$. Cells were harvested in exponential phase (2×10^8 cells/mL) by centrifugation and resuspended in a minimum of buffer, lysed using a French pressure cell and the membranes prepared as described by Futai et al. (1974). ATPase activity was assayed as described by Evans (1969) with corrections for spontaneous hydrolysis of ATP. The reaction was terminated by the addition of trichloroacetic acid. Liberated inorganic phosphate was determined colorimetrically by the method of Rathburn and Betlach (1969). Protein was determined by the method of Lowry et al. (1951). The specific activity of the ATPase preparations was 37.6 ± 5.6 micromoles ATP hydrolysed/(mg protein x hour) at $30^{\circ}C$.

Reconstitution experiments were performed with ATPase as described by Rosen and Adler (1975). ATPase was solubilized using low ionic

strength buffers. This procedure solubilizes the F_1 ATPase with bound annular lipids (Peter and Ahlers, 1975). Stripped membranes were prepared by repeated extraction with the solubilizing buffer and hydrolysed less than 0.20 micromoles of ATP/(mg protein x hour) at $30^{\circ}C$. Reconstituted membranes used for Arrhenius plots hydrolysed about 24 micromoles of ATP/(mg protein x hour) at $30^{\circ}C$.

Arrhenius Plots of Lac Permease and ATPase

During the determination of permease activity, freshly prepared suspensions of cells were held in inhibitor buffer at $0^{\circ}C$. Incubations were performed using individual water baths for a total of 10 to 12 temperature points per plot. During the determination of membrane-bound ATPase activity, fresh membrane preparations were held at $0^{\circ}C$. During the determination of soluble ATPase activity, freshly solubilized enzyme was held at room temperature. For ATPase, fifteen temperatures were assayed concurrently in duplicate using a linear temperature gradient block. Slopes were determined by least square regression analysis. The intercepts were determined mathematically by setting the equations describing each line (above and below the break) equal at log activity and solving for temperature. Coefficients of correlation were determined for each line and ranged from 0.985 to 0.998 for the lac permease and 0.990 to 0.999 for ATPase activity. Omission of the two points nearest the break had little effect on these slopes.

The temperature gradient block used for Arrhenius plots of ATPase consists of an aluminum block 22.5 x 4 x 4 inches. An array of $\frac{1}{2}$ inch

holes (15 x 3) was centered in this block (2.5 inches deep). Pieces (1.5 inches) were cut from each end, routed to form two water channels and fitted with inlet and outlet connections. These were bolted back on the block using a rubber gasket as a seal. A Haake E52 thermostated circulator was connected to one end (hot water) and a Neslab RTE-3 circulator was connected to the other end (cold water/ethylene glycol). The block was encased in 1.0 inch of styrofoam with corresponding holes, mounted in a wooden frame and bolted on an Eberbach reciprocating shaker (Scientific Products, Orlando, Florida). The temperature ranges in the block were established by adjusting the circulator temperatures and the block formed a stable gradient within two hours. The temperature gradient as measured in each well using a NBS standard thermometer did not deviate from linearity with a coefficient of correlation greater than 0.9999. Wells accommodate 13 x 50 mm tubes. Approximately 0.5 mL of water was added to each well to ensure good thermal contact between the block and the sample tube.

Fatty Acid Analysis

Lipids were extracted from cells which had been inactivated with trichloroacetic acid (5%) as described by Kanfer and Kennedy (1963). Fatty acids were transesterified and analysed by gas chromatography (Ingram, 1976). Compositions are reported as percentage of peak area.

Chemicals

All biochemicals used in this study were obtained from Sigma Chemical Company (St. Louis, Missouri). During the course of this

study Cantley et al. (1977) found that the ATP from Sigma used in this report contained a potent inhibitor of (Na,K)-ATPase. This inhibitor, an inorganic vanadium compound, does not effect the Ca^{++}-ATPase activity (Cantley et al., 1977) or bacterial ATPase. This was confirmed in our laboratory using E. coli ATPase with the vanadium free ATP which was also purchased from Sigma.

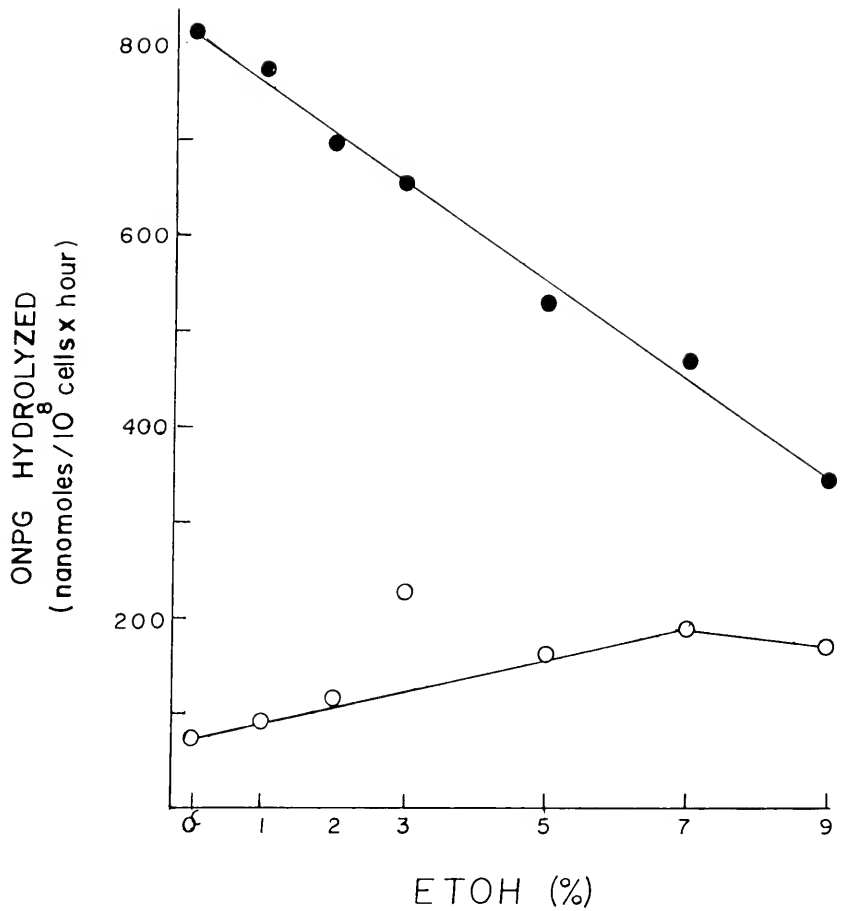
RESULTS

Effect of Ethanol on the Lac Permease

Ethanol causes a dose dependent inhibition of the lac permease (figure 1). Cryptic transport (diffusion and other transport systems) is slightly stimulated by ethanol. These effects are reversible upon removal of ethanol by washing. A variety of other moderately hydrophobic compounds have been shown to induce changes in E. coli lipid composition alalogous to ethanol (Ingram, 1977). Of these, acetone (20g/L), dimethyl sulfoxide (74g/L), methanol (32g/L) and dioxane (20g/L) were tested for their effect on the lac permease. In all cases, these agents inhibited the permease activity. Sullivan et al. (1974) have previously shown that long chain alcohols such as hexanol stimulate permease activity. This stimulation by long chain alcohols was confirmed in strain K1-221. Growth of E. coli in the presence of these long chain alcohols results in changes in fatty acid composition opposite to those caused by ethanol (Ingram, 1976). Chloroform (0.5g/L) and amyl acetate (0.3g/L) induce changes in lipid composition like hexanol (Ingram, 1977) and these agents also stimulate permease activity.

The initial inhibition of permease activity by ethanol is relieved during subsequent growth in the presence of ethanol. Both the permease and β-galactosidase from cells grown in the presence of ethanol exhibit

Figure 1. Acute effect of ethanol on β-galactoside uptake. Lac permease mediated transport, ● ; and cryptic entry, o .


800
600
400
200
ONPG HYDROLYZED
(nanomoles / 10^8 cells x hour)
0
1
2
3
5
7
9
ETOH (%)

the same specific activities as found in control cells. This suggests that the recovery of permease activity does not simply result from the synthesis of new protein. The time required for the recovery of permease activity is directly related to alcohol concentration (figure 2A) and inversely related to the growth rate (figure 3A). Changes in fatty acid composition occur during this recovery period. The time required for the completion of these changes was directly related to alcohol concentration (figure 2B) and inversely related to growth rate (figure 3B). In all cases, permease activity was restored prior to the completion of these changes in bulk lipid composition. Thus the completion of these changes in lipid composition is not essential for the recovery. Protein synthesis in strain K1-221 was inhibited by the addition of chloramphenical (100mg/L) and by threonine starvation. Neither treatment prevented the recovery of permease activity during incubation with ethanol, although a longer time was required (figure 4). The decrease of the permease activity with time in both the ethanol-treated and control cells is presumably due to turnover of permease.

Effect of Ethanol on ATPase

The effect of ethanol on ATPase isolated from E. coli grown at 37° C in the absence of ethanol is dramatically affected by the choice of divalent metal cofactor (figure 5). With Mg^{++}, ethanol caused a dose-dependent stimulation of activity. With Ca^{++}, ethanol caused the opposite effect, a dose-dependent inhibition. This differential effect suggests that ethanol is interacting in some way which alters the shape

Figure 2. Recovery of ethanol-inhibition of lac permease activity during growth concurrent with ethanol-induced changes in fatty acid composition. An exponentially growing culture was split and supplemented with various concentrations of ethanol at time zero. Samples were removed for the determination of fatty acid composition and permease activity. A. Permease activity: no ethanol ○; 16g/L ethanol, ●; 24g/L ethanol, △; and 32g/L ethanol, ▲. B. Fatty acid composition: no ethanol, solid line; 8g/L ethanol, ○; 16g/L ethanol, ●; and 24g/L ethanol, △.

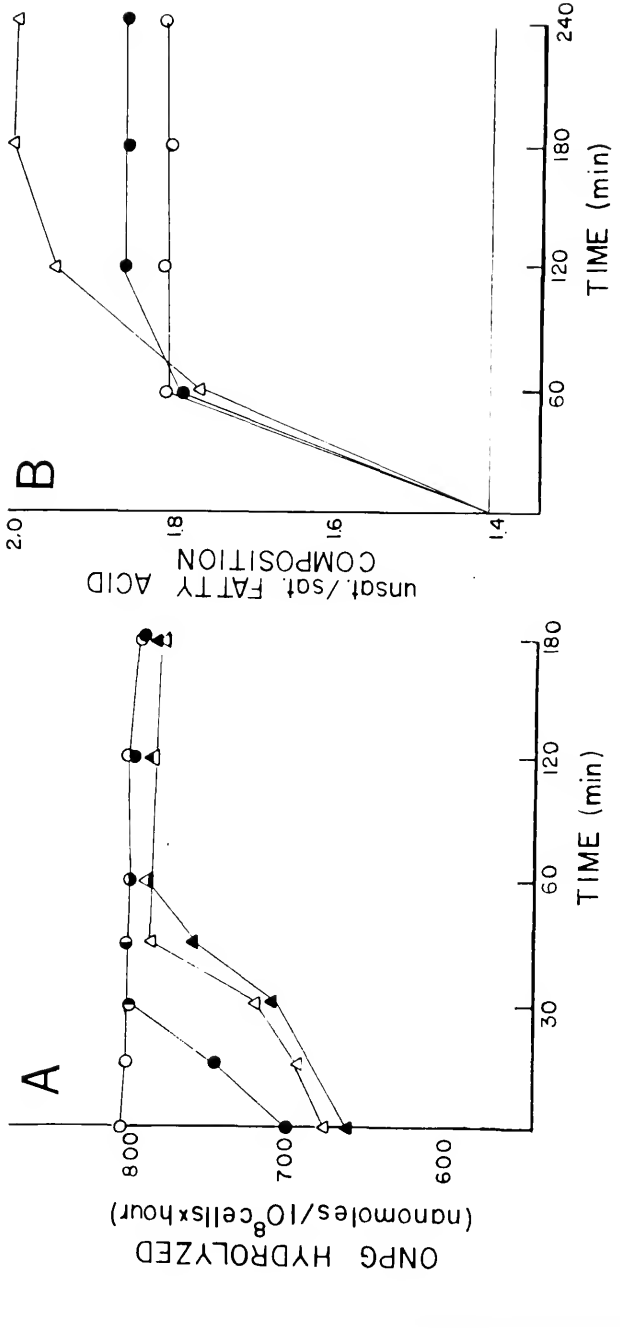
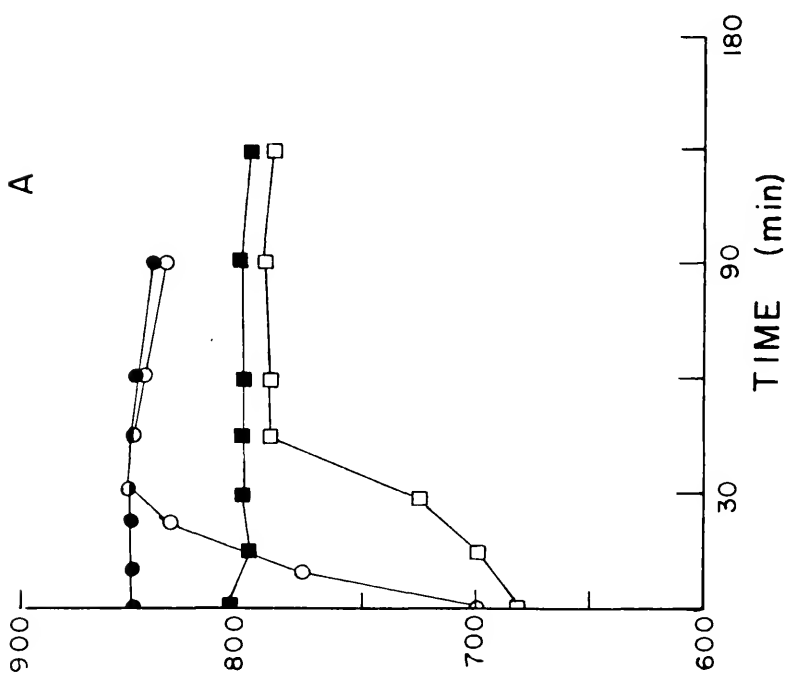

A
ONPG HYDROLYZED
(nanomoles/10⁸cells x hour)
800
700
600
30
60
120
180
TIME (min)
B
unsat./sat. FATTY ACID
COMPOSITION
2.0
1.8
1.6
1.4
60
120
180
240
TIME (min)

Figure 3. Effect of growth rate on recovery of ethanol-inhibited lac permease activity with concurrent ethanol-induced changes in fatty acid composition. A. Permease activity: minimal medium without ethanol, ■; minimal medium with 16g/L ethanol, □; minimal medium with casamino acids and no ethanol, ●; and minimal medium with casamino acids and 16g/L ethanol, ○. B. Fatty acid composition: the effect of ethanol (16g/L) on the fatty acid composition of cells grown in minimal medium, □; and in the same medium supplemented with casamino acids, ○.


A
ONPG HYDROLYZED
(nanomoles/ 10^8 cells x hour)
900
800
700
600
30
90
180
TIME (min)

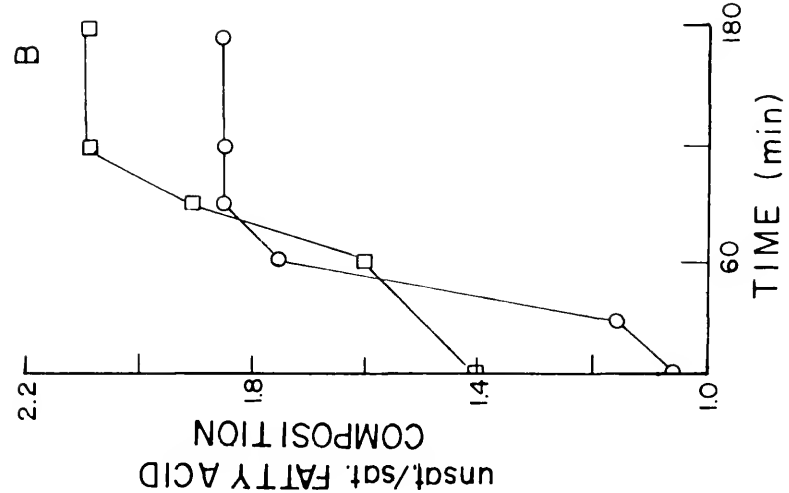

B
unsat/sat. FATTY ACID
COMPOSITION
2.2
1.8
1.4
1.0
60
180
TIME (min)

Figure 4. Recovery of lac permease activity in the presence of ethanol during inhibition of protein synthesis. A. Permease activity in chloramphenicol treated cells: no ethanol, ●; 16g/L ethanol, ○. B. Permease activity in tyrosine starved cells: no ethanol, ●; 16g/L ethanol, ○.

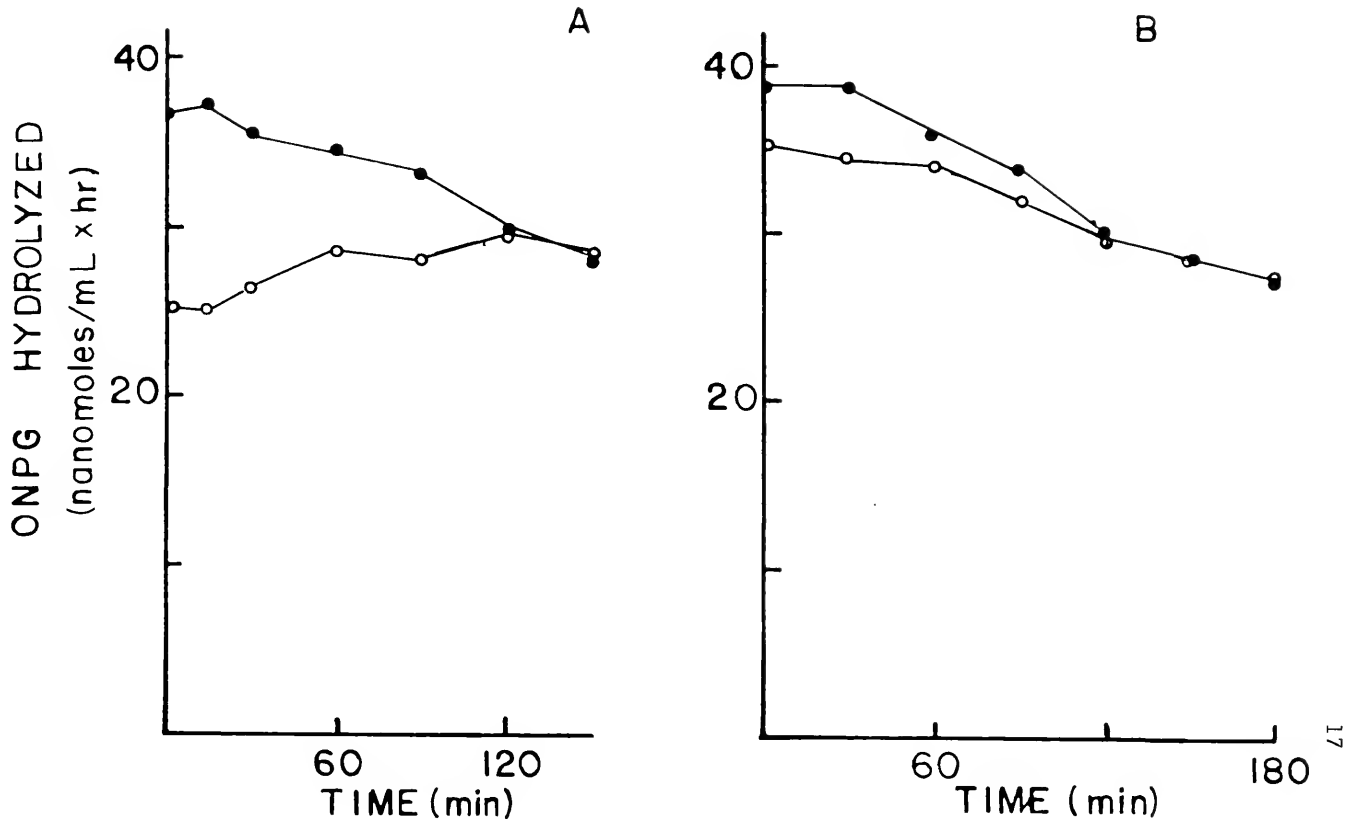
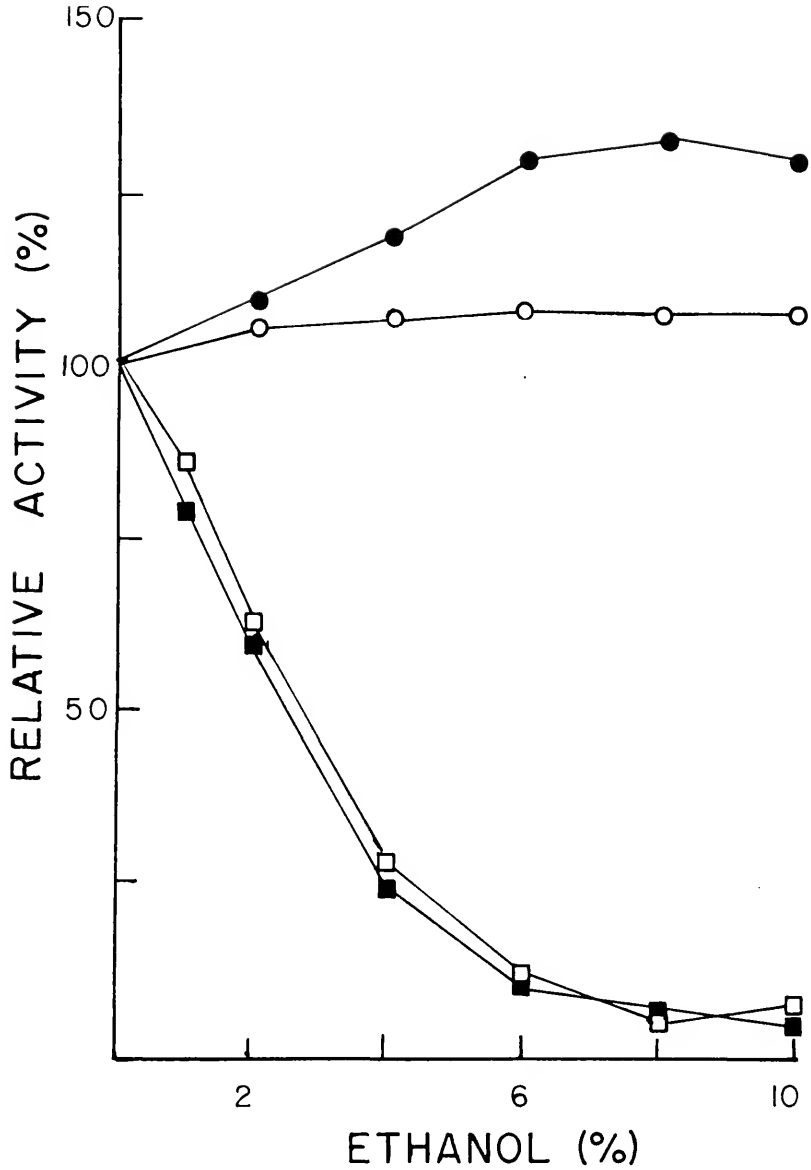

A
B
40
20
ONPG HYDROLYZED
(nanomoles/mL x hr)
60
120
180
TIME (min)
TIME (min)

Figure 5. Effect of ethanol on membrane-bound ATPase from cells grown at 37°C with Mg^{++} or Ca^{++} as a cofactor assayed at 37°C. Control cells with Mg^{++}, ●; control cells with Ca^{++}, ■; ethanol grown cells with Mg^{++}, ○; and ethanol grown cells with Ca^{++}, □.


150
100
50
RELATIVE ACTIVITY (%)
2
6
10
ETHANOL (%)

of the catalytic site of the enzyme. The Km of ATPase was found to be unaltered by the inclusion of ethanol in the assay mixture.

Growth in the presence of ethanol results in changes in the lipid composition which could also influence enzyme function (table I). ATPase from ethanol-grown cells was equally sensitive to inhibition by ethanol using Ca^{++} as a cofactor (figure 5). However, ATPase from ethanol-grown cells was much less stimulated by ethanol using Mg^{++} as a cofactor (figure 5). Thus changes have occurred during growth in the presence of ethanol which partially ameliorate the effect of ethanol on ATPase activity assayed with Mg^{++}.

Growth of E. coli strain CSH-2 at 30° C results in a fatty acid composition similar to that produced during growth at 37° C in the presence of ethanol (table I). When ATPase from cells grown at 30 °C was assayed at 37° C using Mg^{++} as a cofactor, ethanol caused a modest stimulation of activity, intermediate between cells grown without ethanol at 37 °C and 37 °C ethanol-grown cells. This suggests that fatty acid changes may be involved in ethanol resistance and a possible relationship between ethanol and low temperature.

Ethanol has been shown to have little effect on the activity of the Mg^{++} stimulated ATPase of mitochondria from rat kidney when assayed at high temperatures (Hosein et al., 1977). This enzyme was inhibited, however, at lower temperatures (Hosein et al., 1977). The ATPase of E. coli with Mg^{++} as a cofactor is stimulated at 37° C (growth temperature) by ethanol, however a slight inhibition in activity by ethanol was

Table I. The Effect of Ethanol and Fatty Acid Supplements on the Fatty Acid Composition of E. coli Grown at Various Temperatures.

Strain	Growth Temperature	Additive	Fatty Acid Composition (%) 12:0	14:0	16:0	16:1	18:1
Kl-221	30	None	0.8	4.5	38.9	42.2	12.2
Kl-221	30	Ethanol	2.2	5.0	31.9	40.6	18.3
Kl-221	37	None	0.7	4.0	46.0	38.6	11.5
KL-221	37	Ethanol	1.8	4.3	37.3	41.2	15.2
CSH-2	30	None	3.2	3.0	25.9	35.3	32.6
CSH-2	30	Ethanol	3.6	2.3	20.3	33.6	39.0
CSH-2	37	None	3.5	4.2	33.1	36.4	22.9
CSH-2	37	Ethanol	4.0	2.2	27.9	32.2	33.7
WNI	37	None	4.3	8.5	38.8	38.3	5.6
WNI	37	Ethanol	4.4	7.0	40.6	37.2	5.3
TB-4	37	18:1[a]	-	2.0	27.5	21.6	47.3
TB-4	37	16:0[a]	-	1.8	52.3	33.3	11.1

[a] Fatty acid concentration added to the growing culture was 100mg/L in Luria broth without glucose.

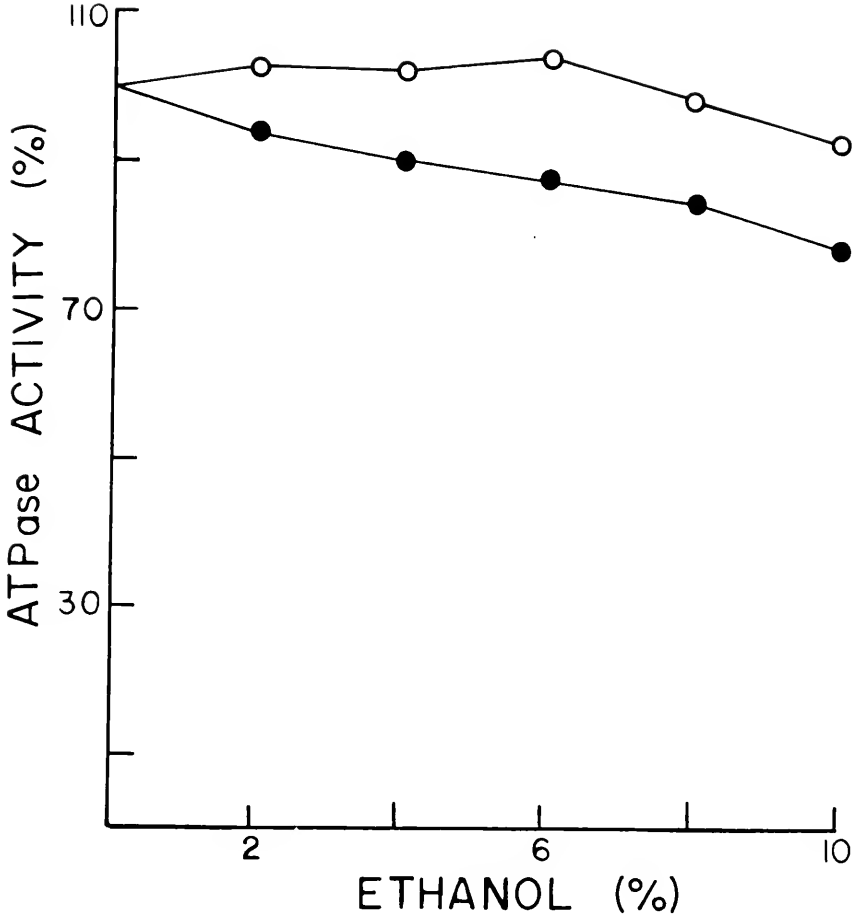
observed when assayed at a lower temperature (figure 6). This inhibition at a lower temperature (15°C) was relieved following growth in the presence of ethanol.

Arrhenius Plots

Arrhenius plots are frequently used to study membrane-bound enzymes (for review see Lenaz et al., 1975). Arrhenius plots of membrane-bound enzymes typically display one or two discontinuities. Massey et al. (1966) have shown a direct correlation between the break temperature in Arrhenius plots and a conformational change in a soluble enzyme. The position of the break in membrane-bound enzymes is presumed to also be due to a conformational change in the enzyme. Break temperatures are dependent upon the specific protein and its bound annular lipids (Hesketh et al., 1976). Only one break was observed in the Arrhenius plots in this report. Based upon previous reports (Linden et al., 1973; Morrisett et al., 1975; and Thilo et al., 1977) the second break would be expected to occur at or below our lowest measured temperatures. In cells grown at 42°C and in cells at 37°C with exogenous palmitic acid (16:0) to boost the saturated fatty acid content, we observed a second break with the ATPase (data not shown).

Arrhenius plots of lac permease have break temperatures which are dependent upon the lipid composition of the membrane (Overath et al., 1971). Cells grown at 30°C contain a lower proportion of saturated fatty acids than cells grown at 37°C (table I). As expected, the break

Figure 6. Effect of ethanol on membrane-bound ATPase from cells grown at 37°C with Mg^{++} as a cofactor and assayed at 15°C. Control cells, ●; ethanol-grown cells, O.


110
70
30
ATPase ACTIVITY (%)
2
6
10
ETHANOL (%)

temperature of the 30° C-grown cells is lower than that for 37° C-grown cells (figure 7A & B). Inclusion of ethanol in the assay mixture resulted in an increase in the break temperature of these Arrhenius plots (figure 7A & B). This increase is similar to the effect caused by an increase in the proportion of saturated fatty acids. The effects of ethanol on Arrhenius plots of lac permease are summarized in table II. From these results it appears that ethanol in some way stabilizes the form of the lac permease with the higher energy of activation, increasing the temperature necessary for the conformation change to the form with the lower energy of activation. Unlike the break temperature, however, the energy of activation of the lac permease appears unaffected by the addition of ethanol with one possible exception (table II), the lower Ea of cells grown at 37° C in the absence of ethanol.

Growth in the presence of ethanol results in changes within the membrane which prevent the ethanol-induced increase in break temperature (figure 7C). Arrhenius plots of ethanol-grown cells assayed in the absence of ethanol display the same break temperature as control cells assayed in the absence of ethanol, despite differences in fatty acid composition (table I). The lipid composition of strain K1-221 grown at 30 °C without ethanol and ethanol-grown cells at 37 °C is very similar (table I). However, the break temperature in these 30 °C-cells is still increased by the inclusion of ethanol. These results suggest that bulk lipid composition alone does not determine break temperature or ethanol resistance.

Figure 7. Arrhenius plots of lac permease. A. Cells grown at 37°C. B. Cells grown at 30°C. C. Cells grown at 30°C in the presence of 16g/L ethanol. Assays were carried out in the absence (●) and presence (○) of 16g/L ethanol.

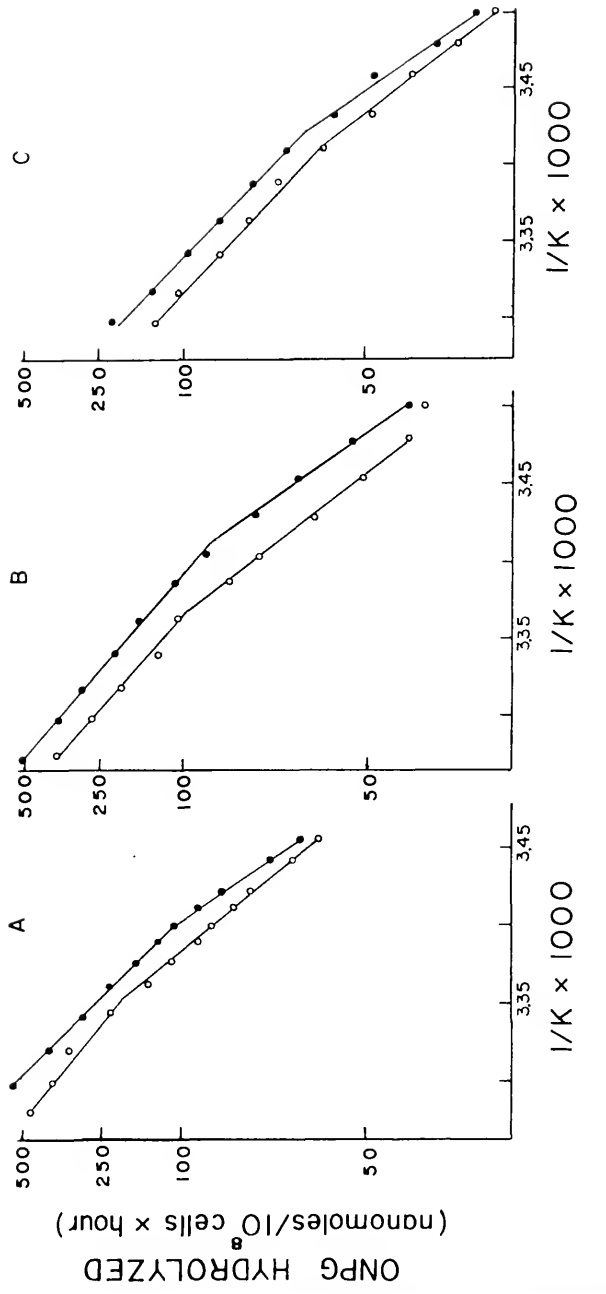

ONPG HYDROLYZED
(nanomoles/10⁸ cells x hour)
A
B
C
500
250
100
50
3.35
3.45
1/K x 1000

Table II. Summary of the Effects of Ethanol (16g/L) on Arrhenius Plots of the lac Permease

GROWTH CONDITIONS Additive	Temperature (°C)	Assay Additive	Break Temperature[a] (°C ± S. D.)[b]	Upper E (Kcal/mole ± S. D.)[b]	Lower E (Kcal/mole ± S. D.)[b]
None	30	None	18.3 ± 0.79 (6)	21.0 ± 2.13	29.4 ± 1.68
		Ethanol	23.6 ± 1.64 (5)	18.0 ± 3.20	30.3 ± 2.16
	37	None	21.2 ± 0.21 (5)	17.4 ± 1.60	29.8 ± 2.13
		Ethanol	23.9 ± 0.57 (7)	16.5 ± 1.19	25.3 ± 1.17
Ethanol (16g/L)	30	None	17.9 ± 0.08 (2)	28.8 ± 2.10	39.1 ± 2.64
		Ethanol	18.6 ± 0.35 (3)	26.4 ± 1.96	30.3 ± 2.16
	37	None	20.7 ± 0.31 (3)	21.7 ± 1.06	29.2 ± 0.96
		Ethanol	21.1 ± 0.20 (3)	19.7 ± 1.50	26.6 ± 1.32

[a]Number within parentheses to the right of S. D. denotes number of replicates.

[b]S. D., standard deviation.

The break temperature in Arrhenius plots of ATPase is less affected by changes in bulk membrane lipid composition than lac permease (figure 8) although lipids are required for ATPase activity (Peter and Ahlers, 1974). As with the lac permease, the inclusion of ethanol in the assay mixture results in an increase in the break temperature of membrane-bound ATPase (figure 8A & B). ATPase from ethanol-grown cells exhibits no such increase in break temperature when assayed in the presence of ethanol (figure 8C). The lipid composition of strain CSH-2 grown at 30°C without ethanol and ethanol-grown cells at 37°C are very similar (table I). However, the break temperature in these 30°C-cells is still increased by the addition of ethanol, suggesting that changes in bulk lipid composition per se do not provide protection against ethanol. The effect of ethanol on Arrhenius plot characteristics of ATPase are summarized in table III. As shown in this table, ethanol did not alter the energies of activation below the break temperature in ethanol-grown cells. The similarities between the effect of ethanol on the break temperature of the lac permease and that of membrane-bound ATPase may reflect a general effect of ethanol on lipid/protein interactions.

ATPase can easily be solubilized by treatment with low ionic strength buffers (Futai et al., 1974). Arrhenius plots of solubilized ATPase exhibit the same break temperature as the membrane-bound form (table III). Similar results were found by Sinerez et al. (1973). Inclusion of ethanol in the assay mixture caused an increase in the break

Figure 8. Arrhenius plots of membrane-bound ATPase from E. coli strain CSH-2. A. Cells grown at 30°C. B. Cells grown at 37°C. C. Cells grown at 37°C in the presence of 16g/L ethanol. Assays were performed in the absence (●) and presence (○) of 16g/L ethanol.

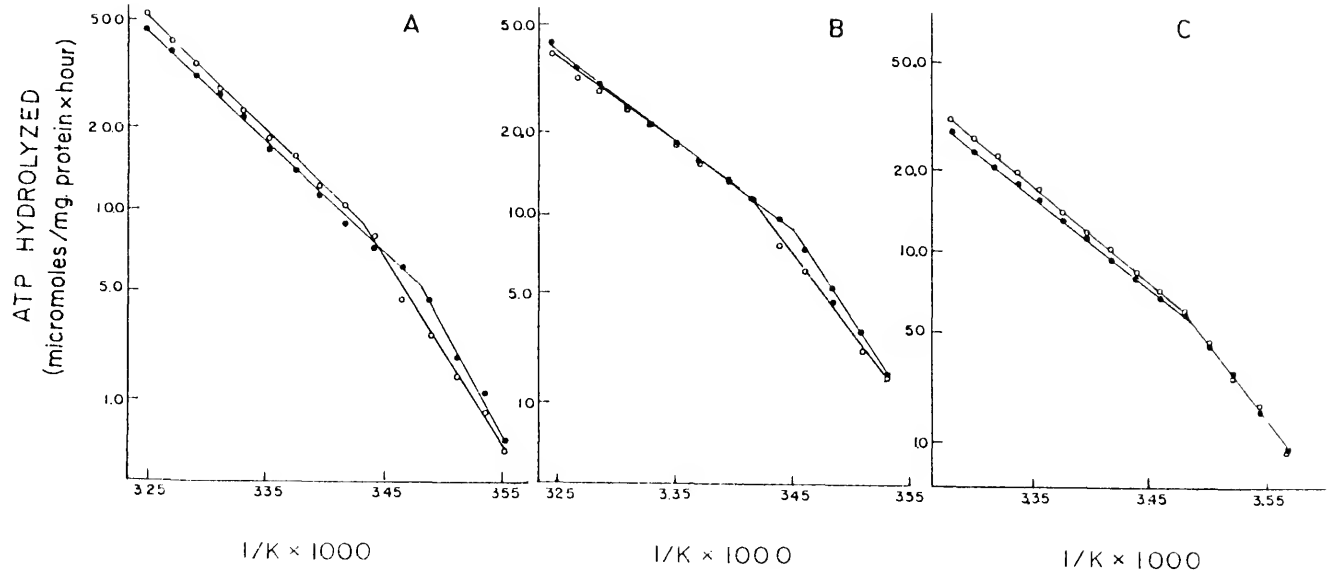

ATP HYDROLYZED
(micromoles/mg. protein × hour)
A
B
C
50.0
20.0
10.0
5.0
1.0
10
50
3.25
3.35
3.45
3.55
1/K × 1000
1/K × 1000
1/K × 1000

Table III. Summary of the Effect of Ethanol (16g/L) on Arrhenius Plots of Membrane-bound and Soluble ATPase

Source of ATPase	Growth Temp. (°C)	Growth Additive	Divalent cofactor	Assay Additive	Break Temperature (°C ± S. D.)[b]	Upper E_a (kcal/mole ± S. D.)[b]	Lower E_a (kcal/mole ± S. D.)[b]
CSH-2 membrane-bound ATPase	30	None	Mg^{++}	None	14.1 ± 0.41 (5)	18.9 ± 0.52	36.6 ± 1.59
				Ethanol	18.0 ± 0.26 (4)	20.1 ± 1.10	32.1 ± 1.36
			Ca^{++}	None	14.3 ± 0.43 (4)	18.5 ± 2.40	34.5 ± 3.28
				Ethanol	17.0 ± 0.66 (4)	18.7 ± 2.01	28.6 ± 3.15
"	37	None	Mg^{++}	None	15.6 ± 0.76 (8)	16.5 ± 0.88	31.1 ± 1.71
				Ethanol	21.3 ± 0.40 (7)	14.9 ± 0.35	25.0 ± 1.78
			Ca^{++}	None	15.6 ± 0.36 (8)	18.6 ± 2.65	34.6 ± 9.04
				Ethanol	19.6 ± 1.09 (6)	18.8 ± 2.02	31.0 ± 2.06
"	30	Ethanol	Mg^{++}	None	13.7 ± 0.22 (3)	19.2 ± 1.02	32.5 ± 1.98
				Ethanol	14.1 ± 0.27 (3)	21.2 ± 0.96	32.1 ± 1.36
			Ca^{++}	None	14.5 ± 0.47 (4)	19.1 ± 2.12	35.1 ± 4.68
				Ethanol	14.8 ± 0.59 (4)	16.3 ± 2.65	29.9 ± 1.68
"	37	Ethanol	Mg^{++}	None	15.0 ± 0.42 (4)	18.9 ± 2.38	33.0 ± 0.92
				Ethanol	14.9 ± 0.59 (3)	21.9 ± 1.95	32.9 ± 1.33
			Ca^{++}	None	16.0 ± 0.19 (5)	17.2 ± 1.93	31.6 ± 3.62
				Ethanol	15.9 ± 0.22 (4)	18.4 ± 2.20	32.6 ± 4.10
CSH-2 soluble ATPase	37	None	Mg^{++}	None	15.7 ± 0.49 (5)	18.0 ± 0.99	31.9 ± 0.42
				Ethanol	19.4 ± 0.21 (4)	20.6 ± 0.64	28.8 ± 0.85
		Ethanol	Mg^{++}	None	15.6 ± 0.21 (4)	23.2 ± 7.07	36.9 ± 3.68
				Ethanol	16.2 ± 0.67 (4)	21.1 ± 4.38	38.8 ± 7.14

[a] Number within parentheses to the right of S. D. denotes number of replicates.

[b] S. D., standard deviation.

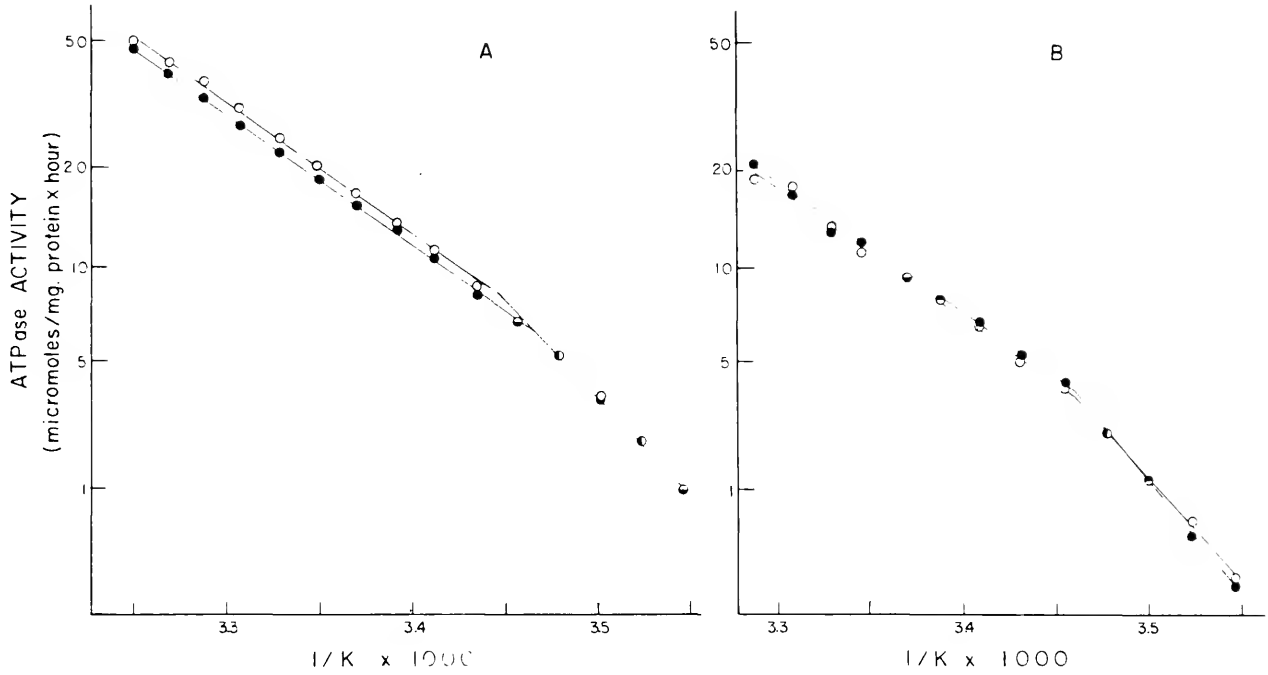
temperature of soluble ATPase (table III) analogous to that observed with membrane-bound ATPase. This indicates a direct effect of ethanol on the soluble ATPase complex. Thus the effect of ethanol on Arrhenius plots is not mediated through the interaction of ethanol with bulk membrane lipids. Soluble ATPase isolated from ethanol-grown cells is also resistant to the effect of ethanol (table III), indicating that bulk membrane is not directly responsible for the protection against ethanol.

E. coli strain WN I fails to alter its lipid composition when grown at different temperatures (Gelmann and Cronan, 1972) or in the presence of ethanol (table I). The membrane-bound ATPase from this organism displays a break temperature in its Arrhenius plot which is increased by the inclusion of ethanol (figure 9A). The break temperature from cells grown in the presence of ethanol is unaffected by ethanol (figure 9B) despite the failure of this strain to alter its bulk fatty acid composition. Again, changes in bulk lipid composition do not appear essential for the amelioration of the ethanol effect on Arrhenius plots of ATPase.

Reconstitution of Membrane-bound ATPase

Membrane-bound ATPase from E. coli can easily dissociate into two parts: the F_0 or transmembrane proton translocating portion and the ATPase active F_1 portion (Vogel and Steinhart, 1976) with bound annular lipids (Peter and Ahlers, 1975). Reconstitution experiments were performed using solubilized F_1 ATPase and ATPase depleted membranes to further examine the importance of bound lipids versus bulk membrane in ethanol resistance. Depleted membranes from

Figure 9. Arrhenius plots of membrane-bound ATPase from E. coli strain WN I. A. Cells grown at 37°C. B. Cells grown at 37°C in the presence of 16g/L ethanol. Assays were performed in the absence (●) and presence (O) of 16g/L ethanol.


ATPase ACTIVITY
(micromoles/mg. protein x hour)
50
20
10
5
1
A
B
3.3
3.4
3.5
1/K x 1000
1/K x 1000

ethanol-grown cells were reconstituted with soluble F_1 ATPase from control cells. The break temperature of this preparation was increased by the inclusion of ethanol (figure 10) indicating that the changes in bulk membrane alone do not provide protection against ethanol. Membranes from control cells were also reconstituted with F_1 ATPase from ethanol-grown cells. This reconstituted ATPase was resistant to the effect of ethanol on break temperature (figure 10B). As a control, ATPase and stripped membranes from ethanol-free cells were reconstituted. This preparation exhibited the same ethanol-induced increase in break temperature as native membranes. Thus the sensitivity and resistance of ATPase to the effects of ethanol on break temperature of Arrhenius plots is a property of the soluble portion containing protein and bound annular lipids, independent of the bulk membrane.

Cooperativity of ATPase Inhibition by Sodium

Membrane-bound ATPase of E. coli is inhibited by sodium ions with Ca^{++} as a cofactor (Evans, 1969). This inhibition is cooperative with a Hill coefficient (n) greater than one (Sinerez et al., 1973). Unlike the break temperature observed in Arrhenius plots, the cooperativity of this inhibition is strictly dependent upon the ATPase being membrane-bound. Cooperativity of sodium inhibition is easily destroyed by treatments such as freezing, assaying at a temperature above or below the growth temperature or by solubilizing the enzyme (table IV).

Figure 11 shows the effect of ethanol on a Hill plot of ATPase. Inclusion of ethanol in assays reduced the Hill coefficient. Dimethyl

Figure 10. Effect of ethanol on reconstituted membrane-bound ATPase from ethanol and control cells. A. ATPase stripped ethanol-grown membranes reconstituted with control F_1 ATPase. B. ATPase stripped control membranes reconstituted with ethanol-grown F_1 ATPase. Assays were performed in the absence (●) and in the presence of 16g/L ethanol, O.

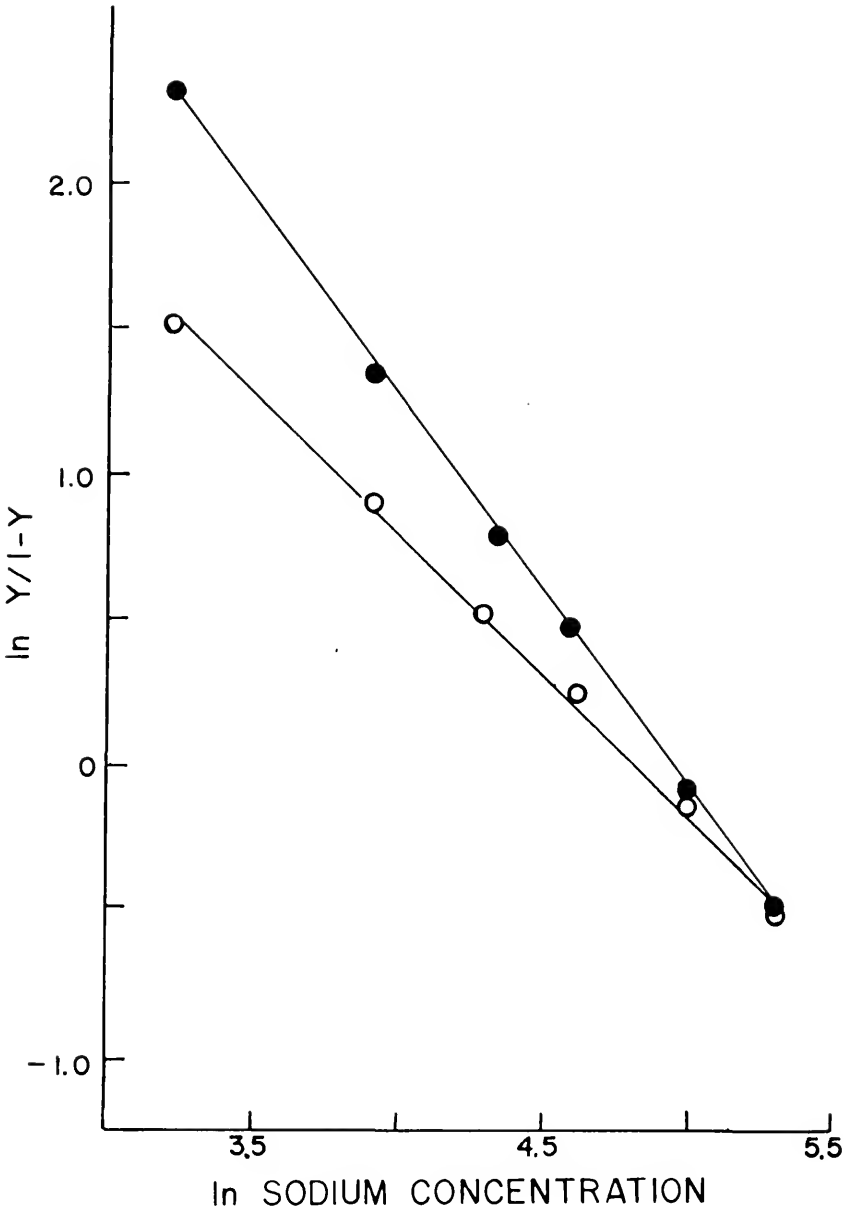
ATP HYDROLYZED
(micromoles/mg. protein × hour)
20
10
5
2
1
A
B
3.30
3.40
3.50
1/K × 1000

Table IV. Effect of a Variety of Physical Treatments on the Cooperativity of Sodium Inhibition of ATPase from *E. coli*[a]

Treatment	Hill Coefficient
None	1.26 ± 0.08 (12)
Freeze and thawed membranes	1.02 (1)
Solubilized ATPase	1.03 ± 0.08 (3)
Assayed at 42°C	1.12 ± 0.04 (2)
Assayed at 30°C	1.09 ± 0.02 (2)

[a] *E. coli* TB-4 was grown at 37°C and assayed at the growth temperature unless otherwise specified.

Figure 11. Hill plots of the sodium inhibition of membrane-bound ATPase. Assays were performed in the absence (●) and the presence (O) of ethanol (16g/L). The sodium concentration was varied over the range of 0-250nM.


2.0
1.0
0
-1.0
ln Y/1-Y
3.5
4.5
5.5
ln SODIUM CONCENTRATION

sulfoxide, pentobarbital and chloropromazine also reduced the co-operativity of sodium inhibition (table V). Cooperativity was not reduced by ethanol in membrane-bound ATPase isolated from cells grown in the presence of ethanol (table V). Additionally, ATPase from ethanol-grown cells was resistant to dimethyl sulfoxide and pentobarbital but remained sensitive to chloropromazine (table V). Pentobarbital (Ingram et al., 1978) and dimethyl sulfoxide (Ingram, 1977) induce changes in fatty acid composition similar to those caused by ethanol while chloropromazine (Ingram et al., 1978) induces nearly opposite changes. The protection provided against other agents by growth in the presence of ethanol suggests that some adaptive change has occurred involving lipids which compensates for the direct physical effect of these agents on ATPase.

To investigate whether changes in bulk membrane fatty acid composition per se are sufficient to afford protection against the direct effect of ethanol on the cooperativity of sodium inhibition, the fatty acid composition of cells was artificially altered. E. coli was grown in the presence of exogenous oleic acid (18:1) to increase the proportion of unsaturated fatty acids within the membrane (table I). Under these conditions, no protection was provided against the effect of ethanol (table VI) in spite of the increased proportion of unsaturated fatty acids. Growth of E. coli with exogenous palmitic acid (16:0) decreased the proportion of unsaturated fatty acids within the membrane (table I) and also had no effect on ethanol sensitivity (table VI). Thus changes

Table V. The Effect of Ethanol and other Membrane Agents on the Hill Coefficients of Membrane-bound ATPase

Assay Additive		HILL COEFFICIENT[a] Control (n ± S. D.)[b]	Grown with Ethanol (16g/L) (n ± S. D.)[a]
None	-	1.26 ± 0.08 (12)	1.28 ± 0.06 (7)
Ethanol	16g/L	0.89 ± 0.14 (9)	1.27 ± 0.09 (8)
Dimethyl Sulfoxide	33g/L	0.98 ± 0.06 (4)	1.24 ± 0.03 (2)
Pentabarbital	20g/L	0.93 ± 0.03 (2)	1.26 ± 0.02 (2)
Chloropromazine	0.2g/L	1.03 ± 0.05 (2)	1.01 ± 0.04 (2)

[a]Number within parentheses to the right of S. D. denotes number of replicates.

[b]S. D., standard deviation.

Table VI. The Effect of a Variety of Treatments on the Protection of the Hill Coefficient Following Exposure to Ethanol

Growth Conditions and Treatments	Hill Coefficient[a] Assayed - Ethanol ($n \pm$ S. D.)[b]	Assayed + Ethanol ($n \pm$ S. D.)[b]
37°C, No Additive	1.26 ± 0.08 (12)	0.89 ± 0.14 (9)
Grown with palmatic acid (100mg/L)	1.29 ± 0.01 (3)	0.89 ± 0.03 (3)
Grown with oleic acid (100mg/L)	1.25 ± 0.02 (2)	1.02 ± 0.06 (2)
Chloramphenicol (100mg/L) without ethanol[c]	1.24 (1)	0.94 (1)
Chloramphenicol with ethanol (16g/L)[c]	1.27 ± 0.03 (2)	1.28 ± 0.04 (2)
Stationary phase cells without ethanol[d]	1.30 ± 0.06 (3)	0.90 ± 0.03 (2)
Stationary phase cells with ethanol (16g/L)[d]	1.25 ± 0.01 (2)	1.26 ± 0.03 (3)
Isolated membranes without ethanol[e]	1.29 ± 0.03 (2)	0.96 ± 0.05 (2)
Isolated membranes with ethanol (16g/L)[e]	1.27 ± 0.07 (3)	1.31 ± 0.03 (4)

[a] Number in parentheses to the right of S. D. denotes the number of replicates.

[b] S. D., standard deviation.

[c] The cells were grown to a density of 2.0×10^8 cells/mL then split into two parts each receiving 100mg/L chloramphenicol. Ethanol (16g/L) was added to one after 30 minutes while the other served as a control. Incubation was continued for 6 hr. prior to harvesting.

[d] The cells were grown to late stationary phase, split into two parts. Ethanol (16g/L) was added to one while the other served as a control. Incubation was continued for 24 hr. prior to harvesting.

[e] Membranes isolated from control cells were split into two parts, one with ethanol (16g/L) the other serving as a control and kept overnight at 37°C prior to the assay.

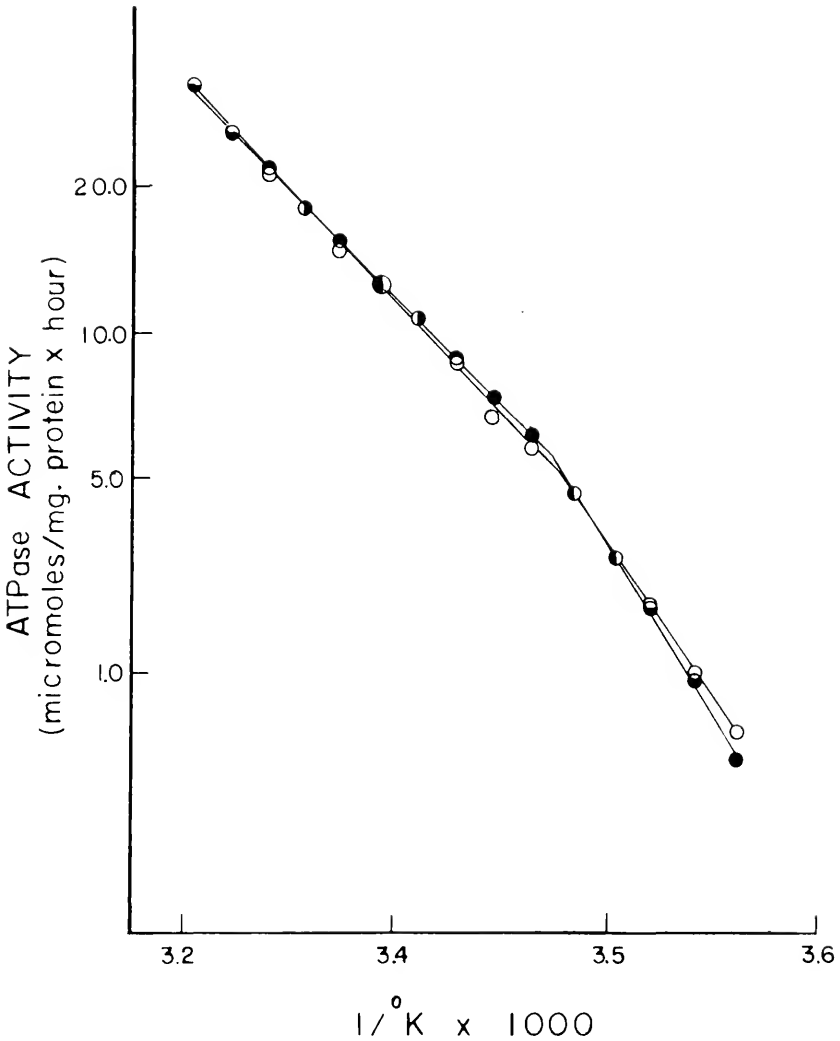
in the bulk lipid composition are not sufficient to provide protection against the effect of ethanol on the Hill coefficient.

The role of growth and new protein synthesis in the acquisition of resistance to ethanol was investigated. Chloramphenicol was added to a culture at a concentration sufficient to stop protein synthesis. The culture was then split into two parts and incubation continued for three hours. One part was supplemented with ethanol (16g/L) while the other served as a control. Membranes were isolated from both of these cultures for the determination of Hill coefficients in the presence and absence of ethanol (table VI). ATPase from the control cells was sensitive to the addition of ethanol while the cells which were exposed to ethanol were resistant to the reduction in the Hill coefficient by ethanol. This indicates that the acquisition of ethanol resistance does not require new protein synthesis. In a similar experiment, a stationary phase culture was split into two parts. Ethanol (16g/L) was added to one part while the other served as a control. Following incubation for an additional 16 hours at 37^{o} C, cells were harvested and the Hill coefficients determined for each in the presence and absence of ethanol (table VI). Stationary cells incubated in the absence of ethanol remained sensitive while those incubated in the presence of ethanol acquired resistance to ethanol. The bulk lipid composition of membranes in stationary phase cells was not altered by the addition of ethanol (table I). This provides further evidence that changes in bulk lipid composition are not essential for the amelioration of ethanol effects.

In Vitro Acclimation of ATPase

Our previous experiments with Hill coefficients and Arrhenius plots indicated that the adaption of membrane-bound ATPase to ethanol does not require new protein synthesis or changes in the bulk lipid composition. This hypothesis was further examined in vitro. Isolated membranes were incubated for 12 hours at 37° C in the presence and absence of ethanol. The cooperativity of sodium inhibition was protected from the ethanol effect following prior incubation in the presence of ethanol (table VI), analogous to the results obtained in vivo with whole cells. Likewise, the Arrhenius plots of the membrane-bound ATPase incubated for 12 hours with ethanol were resistant to the inclusion of ethanol (figure 12). Similar experiments, in vitro, were attempted with soluble ATPase (7 & 12 hour incubations). Storage of the soluble ATPase in ethanol, however, resulted in Arrhenius plots which did not exhibit a distinct discontinuity in their slopes. The adaptation of membrane-bound ATPase in vitro indicates that resistance can be acquired by a rearrangement of ATPase with existing membrane components. The failure of soluble ATPase to maintain its native thermodynamic properties following prolonged incubation in the presence of ethanol suggests that this rearrangement may involve other components in addition to the bound lipids.

Figure 12. Arrhenius plots of membrane-bound ATPase previously exposed to ethanol (16g/L) *in vitro*. Assays were performed in the absence (●) and presence (○) of 16g/L of ethanol.


ATPase ACTIVITY
(micromoles/mg. protein x hour)
20.0
10.0
5.0
1.0
3.2
3.4
3.5
3.6
1/°K x 1000

DISCUSSION

Our results indicate that growth in the presence of ethanol results in changes which confer resistance to the effects of ethanol on two membrane-bound enzymes. The lac permease is inhibited in vivo by the initial addition of ethanol to cells grown without ethanol. During growth in the presence of ethanol, this inhibition is ameliorated (figure 2). Membrane-bound ATPase (Mg^{++}) from cells grown without ethanol is inhibited in vitro by the addition of ethanol at 15° C and stimulated by the addition of ethanol at 37° C (figures 5 & 6). Again ATPase preparations from cells grown in the presence of ethanol are substantially resistant to both effects. Arrhenius plots of lac permease and ATPase exhibit a discontinuity, the position of which is shifted to an elevated temperature by the inclusion of ethanol in the assay mixture (figures 7 & 8). Enzyme preparations from cells grown in the presence of ethanol are resistant to this effect of ethanol. Ethanol eliminates the cooperativity of Na^{+} inhibition of ATPase from control cells. Again, preparations from cells grown in the presence of ethanol are resistant to ethanol. Thus adaptive changes occur during growth in the presence of ethanol which ameliorate the direct effect of ethanol on these membrane functions.

The effect of alcohols on biological systems appears to result from their interaction at hydrophobic sites (Ingram, 1976; and Seeman, 1972).

These hydrophobic sites need not be limited to the membrane lipid bilayer but may also include the hydrophobic regions of proteins and their annular lipids. Following prolonged exposure to ethanol two types of membrane adaptation are frequently observed. The first involves changes in physical properties of membrane-bound enzymes (Hosein et al., 1977; and Israel and Kuriyama, 1971) and the second involves changes in the bulk lipid composition of membranes (Ingram, 1976; Littleton and John, 1977; and Miceli and Ferrel, 1973).

Membrane-bound enzymes are affected by changes in their lipid environment (Esfahani et al., 1971; Kimelberg and Papahadjopoulas, 1972; and Mavis and Vagelos, 1972). The interaction of drugs like ethanol with the membrane alters the physical properties of the membrane and affects the activities of membrane-bound enzymes (Lee, 1976). The changes in bulk lipid composition following growth in the presence of ethanol (table I) have been postulated as part of an adaptive response to compensate for some of these effects (Ingram, 1976).

We have examined the role of the lipids in the adaptation of membrane-bound enzymes. Our results with membrane-bound and soluble ATPase (table III) indicates that the effect of ethanol on Arrhenius plots of this enzyme is mediated through a direct interaction of ethanol with the enzyme and perhaps annular lipid rather than through changes in bulk membrane. Reconstitution experiments indicate that sensitivity or resistance to ethanol are determined within the solubilized portion containing bound lipids, independent of bulk membranes. The

adaptation of ATPase to ethanol in E. coli strain WN I (figure 9) as well as the adaptation of stationary cells (table VI) and isolated membranes (figure 12 and table VI) to ethanol indicates that the bulk lipid changes are not essential for the adaptation of membrane-bound enzymes to ethanol. However, changes in lipid composition may be of significance for other cellular function in vivo.

Membrane-bound enzymes have been shown to exhibit some selectivity for particular phospholipids from their environment which allows maximal enzymatic activity (Warren et al., 1975). Indeed, the isolation of different regions of the membrane has provided direct physical evidence that membrane proteins may partition between specific lipid domains in vivo (van Heerikhuisen et al., 1975; and Letellier et al., 1977). These associations with particular annular lipids presumably maintain the enzyme in the most thermodynamically favored configuration. Thus membrane-bound enzymes appear to behave somewhat like a cartesian diver in that they appear to select the proper environment (i.e. annular lipid composition) to maintain the thermodynamically favored state. The intercalation of ethanol results in changes in the membrane environment. In the presence of ethanol, membrane-bound enzymes may exchange their annular lipids for more unsaturated species to maintain the most thermodynamically favored state.

This cartesian diver model explains how membrane-bound enzymes could adapt in vitro to ethanol. The selection of a new environment by membrane-bound enzymes would only require the proper unbound lipids

within the membrane with which the enzyme could reassociate. In this model, the increase in unsaturated fatty acids following growth in the presence of ethanol could maintain the proper microviscosity in the bulk lipid bilayer which would have become more saturated in nature due to the removal of unsaturated phospholipid species by this protein/lipid reassociation. Alternatively, an increase in unsaturation of molecular species could increase the proportion of the lipid environment with the physical properties which optimize enzyme function. This model suggests that cells with naturally higher levels of unsaturated fatty acids in their bulk lipids would be capable of coping with ethanol better than cells with lower levels of unsaturated fatty acids. Recently we have isolated fifteen mutants of E. coli which are resistant to ethanol all of which contain higher proportions of unsaturated fatty acids than the parental strain when grown in the absence of ethanol.

This model for the development of ethanol tolerance for membrane-bound enzymes may be related to the observed consequence of prolonged alcohol consumption. An initial acute tolerance occurs following several hours of exposure to ethanol. This tolerance may be due to rearrangement of membrane proteins with existing lipids as described in our model. Chronic tolerance and its associated dependence could be due to both this rearrangement and the ethanol-induced changes in membrane composition (Ingram et al., 1978; and Littleton and John, 1977). These lipid changes need not be limited to phospholipids and

fatty acids. Other membrane components such as cholesterol and carotenoids may also be involved.

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BIOGRAPHICAL SKETCH

Benjamin Fisher Dickens Jr. was born on August 1, 1951, in Jacksonville, Florida. He lived in Fernandina Beach, Florida, with his parents, Dr. and Mrs. B. F. Dickens, from 1951 until 1969 when he graduated from Fernandina Beach Senior High School. In September 1969 he began attending college at the University of Florida in Gainesville, Florida. He obtained his Bachelor of Science in June 1973 and in March 1976 he obtained his Master of Science from the University of Florida. He married Bonnie Estelle Quattlebaum on July 1, 1972, and their son, Brian Fisher, was born on April 21, 1975. Benjamin is a candidate for the degree of Doctor of Philosophy in December 1978.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lonnie O. Ingram, Chairman
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward M. Hoffmann
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles M. Allen Jr.
Associate Professor of Biochemistry

This dissertation was submitted to the Graduate Faculty of the Department of Microbiology and Cell Science in the College of Liberal Arts and Sciences and to the Graduate council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1978

Dean, Graduate School